Exhibit E

Apoptosis: a different type of cell death

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ABSTRACT **Apoptosis is a type of cell death that plays an important role in early development and growth of normal adult tissues. It is regulated by physiological stimuli and is present in many species and tissues. The main morphological characteristics are nuclear fragmentation and cellular breakdown in apoptotic vesicles. Internucleosomal DNA fragmentation is an important biochemical feature that is the result of a yet to be isolated endonuclease activity. Experiments using RNA and protein synthesis inhibitors suggest the presence of either intracellular repressors or inducers of apoptosis.—Gerschenson, L. E.; Rotello, R. J. Apoptosis: a different type of cell death. *FASEB J.* 6: 2450–2455; 1992.**

Key Words: apoptosis • programmed cell death • internucleosomal DNA fragmentation

CELL DEATH HAS TRADITIONALLY been a research endeavor pursued mostly by pathologists. The reason is that the cellular basis of disease—cell injury and repair, which in extreme cases result in cell death and harm to the organism—are classical realms of those medical researchers. However, there is now a general research interest in a type of cell death called apoptosis, which is usually not the result of injury. It is not reversible; it may not be harmful to the host and it may even be necessary for normalcy. This type of cell death is quite different from the necrosis usually studied, which is always the result of injury.

HISTORY AND TERMINOLOGY

The death of cells in the normal development of vertebrates and invertebrates was described originally by Glucksmann in 1951 (1) and by Saunders in 1966 (2). Kerr in 1965 (3) described different types of liver cell death after portal vein branch ligation. Patches of confluent necrosis were seen early, but during the following weeks a different type of cell death was observed in scattered individual hepatocytes that had shrunken nuclei, without any evidence for lysosomal rupture or inflammation. In 1971, the same author described the ultrastructure of what he called "shrinkage necrosis." The nuclear mass or masses were found to be membrane-enclosed bodies containing pieces of condensed chromatin and well-preserved organelles (4). Those morphological changes were also described in a variety of animal tisues under physiological stimulation (5). It was then proposed by Kerr and Searle (6) to name it "apoptosis" (from the Greek: falling off), suggesting it might play an role opposite to that of mitosis. A group of researchers recently attempted to better define the term apoptosis because of confusion in terminology (7). The term apoptosis is sometimes considered synonymous with programmed cell death and it somehow implies a lethal genetic program, which still has not been demonstrated as discussed below. A better term for the deletion of cell populations seen in embryonic development would be programmed cell deletion, which usually occurs by apoptosis. The adjective programmed would then allude to a developmental program, without implications for casual changes in gene expression. The term apoptosis then should be confined to a specific form of cell death that plays an important role in development and growth regulation.

MORPHOLOGICAL CHARACTERISTICS

In contrast to necrosis, there is no marked inflammatory reaction and organelle swelling in apoptotic cells. Earlier nuclear compaction and cytoplasmic condensation are observed, followed by breakdown of the nucleus into discrete fragments. Cells break down into membrane-bound apoptotic bodies or vesicles in which cytoplasmic organelles appear to be intact and most of the apoptotic bodies have a nuclear component. Those bodies are either taken up by adjacent cells in epithelia or they may be sloughed off. Macrophages sometimes play a role in the removal of the apoptotic bodies. This type of cell death occurs in individual cells in an asynchronous fashion, which also differentiates it from necrosis (5) (**Table 1**).

BIOCHEMICAL CHARACTERISTICS

The best-defined biochemical event in apoptosis involves nuclear DNA. Double-strand cleavage of DNA is observed at linker regions between nucleosomes. Those 180-200 base pair fragments are readily shown by agarose gel electrophoresis of DNA and it appears as a typical ladder pattern, whereas in necrosis the DNA breakdown is random and seen as a smear after electrophoresis. The internucleosomal DNA fragmentation is almost always detected when the morphological changes of apoptosis are recognized (8, 9) (**Fig. 1**).

To date, a specific endonuclease or internucleosomal cleavage activity has not been purified, but evidence from various experimental systems suggest increased activity by either activation or synthesis de novo (10, 11). Although internucleosomal DNA cleavage has not been demonstrated to be the lethal event in apoptosis, cells are probably not capable of repairing these numerous double-strand breaks and eventually die.

The phenomenon of DNA fragmentation may not always correlate with vertebrate apoptotic morphology, or with the morphological observations of cell death seen during development of lower invertebrates. In mammalian cells, for example, the same batch of killer T lymphocytes induced similar apoptotic changes in different radiolabeled target cells, Raji and P185 cells. Internucleosomal doublestrand breaks are detected only in the Raji lymphoma cells, whereas P185 mastocytoma cells contained only single-strand breaks

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TABLE 1. *General differences between apoptosis and necrosis*[a]

Characteristics	Apoptosis	Necrosis
Stimuli	Physiological	Pathological (injury)
Occurrence	Single cells	Groups of cells
Reversibility	No (after morphological changes)	Yes (up to the point of no return)
Adhesions between cells and to BM	Lost (early)	Lost (late)
Cytoplasmic organelles	Late stage swelling	Very early swelling
Lysosomal enzyme release	Absent	Present
Nucleus	Convolution of nuclear outline and breakdown (karyorrhexis)	Disappearance (karyolysis)
Nuclear chromatin	Compaction in uniformly dense masses	Clumping not sharply defined
DNA breakdown	Internucleosomal	Randomized
Cell	Formation of apoptotic bodies	Swelling and later disintegration
Phagocytosis by other cells	Present	Absent
Exudative inflammation	Absent	Present
Scar formation	Absent	Present

[a]This table summarizes in broad terms the differences between apoptosis and necrosis. There are only a few exceptions to the general concepts presented in it.

(12, 13). With respect to invertebrate cell death, Lockshin et al. (14, 15) were unable to detect DNA fragmentation in tobacco hornworm liabial glands at specific instar stages of development. These investigators have not detected the DNA ladder in limb interdigital cell death either (14, 15). Therefore, it appears that DNA cleavage may not be a universal event. However, it is present in most cells undergoing apoptosis and it provides a useful biochemical marker to characterize the biology and kinetics of apoptosis in different systems (5) (**Fig. 2**).

Wyllie (8) was the first to demonstrate that in the presence of a synthetic glucocorticoid, the chromatin of rat thymocytes was cleaved into a series of fragments similar to ones observed if isolated nuclei are digested with micrococcal nuclease. At this time, Hewish and Burgoyne (16), who were involved in chromatin studies unrelated to cell death, demonstrated the presence of similar DNA products in isolated liver nuclei incubated with Ca^{2+} and Mg^{2+}. In subsequent studies by Cohen and Duke (10) endonuclease activity was activated by the same cations in isolated thymocyte nuclei, but the half-life of this activity appeared to be very short. Although immature thymocytes are a valuable model to study cell death, one must consider that approximately 20% of these cells are dying at any time, without added exogenous apoptotic inducers (17).

Those early endonuclease studies supported the idea that Ca^{2+} mobilization was the key cation to activate the death program and eventually internucleosomal cleavage. More recent work on thymocyte cell death by McConkey et al. (18, 19) showed that increased levels of Ca^{2+} in immature thymocytes could activate DNA fragmentation, which was suppressed by the activation of protein kinase C. Tomei et al. (20) have also shown that the protein kinase C activator TPA can prevent apoptosis in C3H-10T1/2 cells after serum removal or exposure to ionizing radiation, which supports the idea of suppressing apoptotic cell death through activation of protein kinase C. Those mechanisms of cell death suppression are unknown, but one could envision a cascade of kinase or phosphatase activations resulting from various apoptotic signals. In contrast, apoptosis (internucleosomal DNA cleavage) in human CEM lymphocytes appears to be mediated by a non-Ca^{2+}-requiring mechanism (or mechanisms) (21). In those studies, Alnemri and Litwack (21) suggest that the topoisomerase II inhibitor novobiocin directly and glucocorticoids indirectly mediate lymphocyte death by unmasking or exposing internucleosomal DNA regions that are accessible to non-Ca^{2+}-dependent endonucleases. Thus, calcium seems to have a role in apoptosis in certain experimental systems, but it may not be the only mechanism for activation of apoptosis.

A wide spectrum of agents have been shown to induce apoptosis in cells, particularly thymocytes (**Table 2**). The list of apoptosis-inducing agents no doubt will continue to expand along with the growing interest in this type of cell death. One could speculate that specific cell types may be stimulated to die through apoptosis by multiple pathways, particularly thymocytes. For example, cells can initiate apoptosis in the presence of RNA and protein synthesis inhibitors, probably because mRNA stability (22, 23) is altered, which could be a signal for the cells to die. Drugs that alter

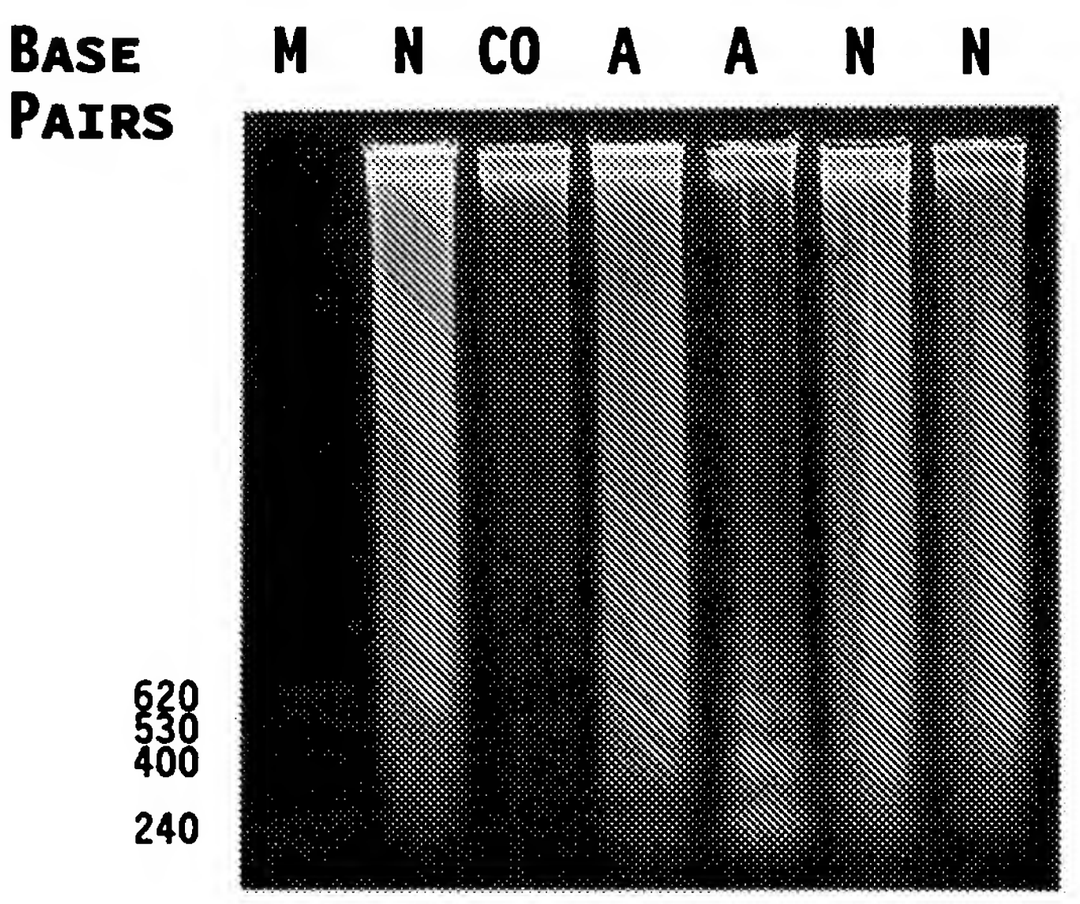

Figure 1. Fragmentation of DNA. Ethidium bromide-stained agarose gel demonstrating fragmentation of endometrium DNA from rabbits subjected to different treatments. Lane M shows molecular weight markers, lane CO shows lack of cell death and DNA degradation, lanes N show the pattern of DNA degradation in necrosis, and lanes A show the characteristic DNA ladder pattern fragmentation of apoptosis.

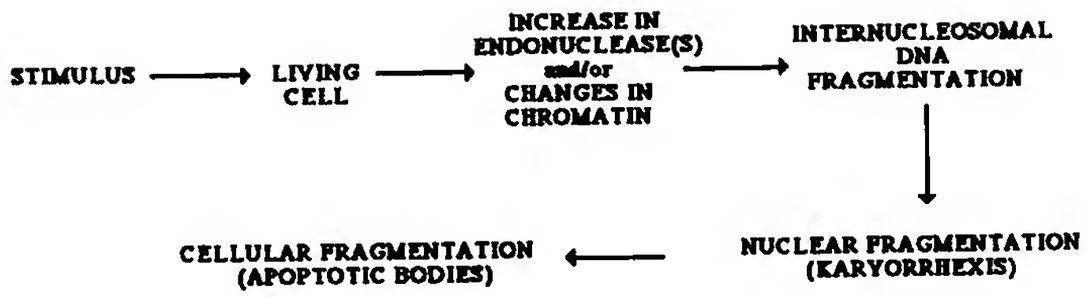

Figure 2. Sequence of events in cells undergoing apoptosis.

chromatin structure such as topoisomerase-II inhibitors may affect DNA conformation and accessibility, which could allow apoptotic endonuclease (or endonucleases) to initiate DNA fragmentation (Table 2).

Occurrence characteristics

Apoptosis has been described in normal embryonic development and metamorphosis (1, 2). Larval organ regression during metamorphosis (24), deletion of interdigital areas (25), retina development (26), and palate fusion (27) are good examples of cell populations deletion by apoptosis in development. In the nematodie *Caenorhabditis elegans*, several genes have been described that regulate apoptosis in different cell types (28–30). Apoptosis in embryogenesis has been shown to be regulated by hormones and local factors (31).

Withdrawal of hormones also results in atrophy of hormone-dependent tissues and apoptosis has been found to be involved in this process. Prostate (32), adrenal cortex (33), and endometrium (34, 35) apoptotic cell death have been described in their involution due to hormone withdrawal.

Apoptosis has also been described in the immune system, where it appears to play an important role in deletion of certain lymphocyte clones (36). It has also been observed in lactating mammary gland regression (37), ovarian follicle atresia (38), neonatal adrenal cortex (39), megakaryocytes (40), leukocytes (41), etc. Lymphocytes can induce apoptosis in target cells. T cells (42), K cells (43), and NK cells (44) have been described to do so. The presence of apoptosis in neoplastic tissue has also been described (6, 45), but its regulation has not yet been studied.

Regulation

The role of apoptosis in adult tissues has not been clarified. The term "programmed cell death" was used to imply the existence of a gene or set of genes that would code for protein (or proteins) having a lethal effect (suicide?) in the same cells harboring the genes. Work with *C. elegans* has shown the existence of several genes that regulate autonomous cell death (28, 29, 30). However, potential homologs still have to be identified in mammalian systems. In general, most examples of programmed cell death showed regulation by factors or hormones. The ecdysteroids, a class of steroids controlling insect metamorphosis, have been described as mediators of neurons and muscle death in the moth *Manduca sexta* (46, 47). Furthermore, a causal relationship has been described between mutations in one region of the decapentaplegic gene

TABLE 2. *Stimuli for apoptosis in different systems (selected list)*[a]

Stimuli	Cell type	Internucleosomal DNA fragments	Apoptotic nuclear and cytoplasmic morphology	Ref
ATP	Thymocytes, lymphoma cell line	Yes	Yes	(61)
Actinomycin D	Leukemia cell line, primary cultures of rabbit endometrium	Yes	Yes	(54, 56)
A23187 Ca^{2+}-Mg^{2+} ionophore	Thymocytes, prostate organ culture	Yes	Yes	(62, 63)
Cytochlasin B	T cell lymphoma line	Yes	?	(64)
Calcium	Immature thymocytes	Yes	Yes	(10, 18)
Cycloheximide	HL-60, 1° cultures of rabbit endometrium	Yes	Yes	(54, 56)
Anti-CD3/T-cell receptor antibody	Immature mouse thymocytes	Yes	Yes	(65)
Epipodophyllo-toxins	Thymocytes	Yes	Yes	(66)
Gliotoxin	Macrophages, T blasts	Yes	Yes	(57)
Glucocorticoids	Thymocytes, lymphoma cell lines	Yes	Yes	(8, 10)
Hyperthermia	Lymphoma, mastocytoma cell lines	Yes	Yes	(67)
Irradiation (soft beta or gamma)	Thymocytes, mouse fibroblasts	Yes	Yes	(6)
Lymphotoxin	Target cells	Yes	Yes	(68, 69)
RU 486	Rabbit uterine epithelium	Yes	Yes	(54)
TCDD	Rat immature thymocytes	Yes	Yes	(18)
Tumor necrosis factor	Target cells	Yes	Yes	(69, 70)
TGF-β1	Cultured rabbit endometrial cells	Yes	Yes	(55)

[a]The presence of internucleosomal DNA fragments and apoptotic morphology were considered to be possible if the investigators showed either a gel demonstrating DNA fragmentation or micrographs of apoptotic cells, or discussed both of them as being present.

of *Drosophila*, which codes for a transforming growth factor β-1 homolog and apoptosis in adult appendages (48).

In mammalian adult tissues, apoptosis can be regulated by either steroid or peptide hormones. Glucocorticoid addition induces it in lymphoid cells (49), whereas removal of IL 2 has a similar effect on IL 2-dependent cells (50). Lack of erythropoietin accelerates apoptosis in bone marrow cells (51), removal of androgens results in apoptosis in prostatic epithelium (52, 53), and progesterone withdrawal elicits it in uterine epithelial cells (34, 35, 54). Transforming growth factor β-1 has been shown to induce apoptosis in primary cultures of the latter tissue (55).

Therefore, the exogenous modulation of apoptosis has been extensively shown, suggesting that the anthropomorphic concept of "suicide" implied in the term programmed cell death may not be correct. It appears that apoptosis is the result of stimuli that trigger responses resulting in a characteristic type of cell death. Is it possible that those exogenous stimuli may trigger the de novo synthesis of intracellular proteins, which could be lethal to the same cells? Research supporting this proposition has shown that administration of RNA and protein synthesis inhibitors results in suppression or delay of apoptosis (10, 18), implying that an intracellular positive signal may be necessary. However, it has also been shown that those inhibitors can induce apoptosis (56) and that gliotoxin induced apoptosis in macrophages while inhibiting protein synthesis (57). Our own research on apoptosis regulation in rabbit endometrium has shown that there is a lag period of several hours after triggering apoptosis (54), suggesting the probable need for RNA and/or protein synthesis. However, studies using primary cultures of that same tissue showed that several RNA and protein synthesis inhibitors induce apoptosis in cells cultured with serum, which suppresses that type of cell death (R. J. Rotello, R. C. Lieberman, and L. E. Gerschenson, unpublished results). Therefore, experimental evidence until now appears to be equivocal. There may be an intracellular short half-life repressor of apoptosis and apoptosis induction could result in its inactivation. On the other hand, there may be an intracellular inducer of apoptosis whose synthesis or activation could be turned on by appropriate stimuli. It is also possible that apoptosis repressors and inducers could coexist in cells, and apoptosis regulation could result because of differential half-lives or interactions (**Fig. 3**).

The role of apoptosis in normal growth regulation has not been studied extensively, but research using rabbit uterine epithelium has shown that cell proliferation and apoptosis

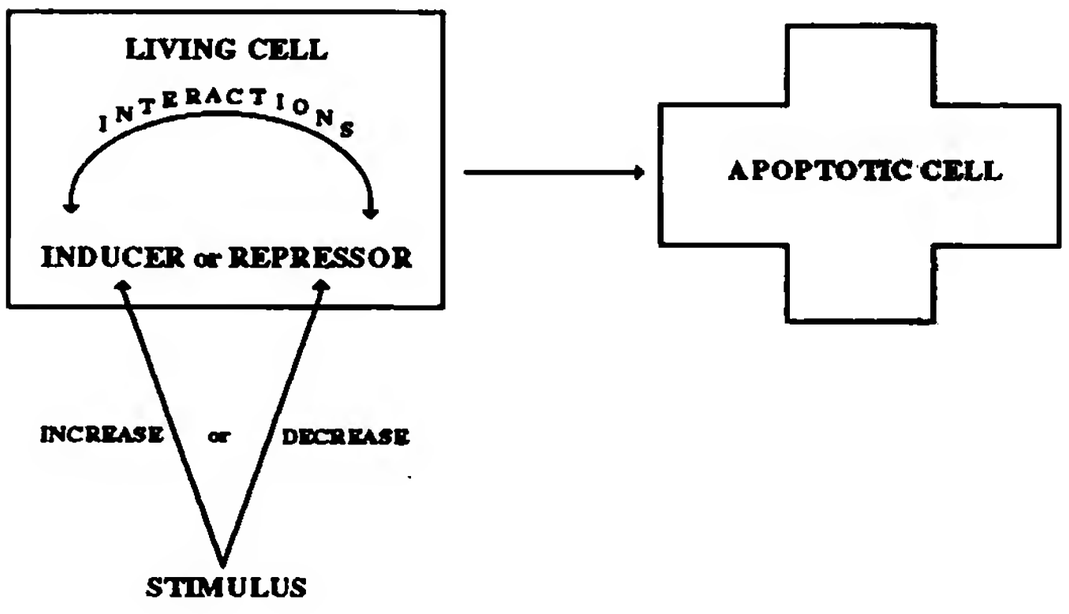

Figure 3. Apoptosis regulation. Hypothetical model proposing the existence of apoptosis inducers and/or repressors in cells.

REGULATORY ROLE	END RESULT
IN DEVELOPMENT	PROGRAMMED DELETION of CELL POPULATIONS
IN ADULT TISSUES	GROWTH MODULATION = CELL PROLIFERATION *minus* APOPTOSIS

Figure 4. Roles of apoptosis. The two main functions of apoptosis are in development and growth regulation.

are regulated in an inverse and coordinated fashion by progesterone in animals and by transforming growth factor β-1 as well as serum in primary cultures (54, 55; R. J. Rotello, R. C. Lieberman, and L. E. Gerschenson, unpublished results). It has also been shown recently that Epstein-Barr virus latent proteins activate B cell proliferation and decrease apoptosis (58). The effect appears to involve the cellular oncogene bcl-2 (59). The products of the tumor suppressor gene p53 have been described to be involved in arresting growth by increasing apoptotic activity (60). Therefore, evidence shows that cell proliferation and apoptosis may be subject to coordinated but inverse regulation (**Fig. 4**).

CONCLUDING REMARKS

The previous brief discussion of apoptosis characteristics shows clearly its wide distribution in species and tissues, as well as the existence of multiple effectors. However, a reductionist approach would require a single mechanism of action. Originally it was thought that in cells there may be a gene or set of genes (death genes?) that my be programmed to be expressed spontaneously at a given time resulting in the synthesis of lethal products, which would then kill the cells harboring them (suicide?). The work using *C. elegans* provides the strongest support of that hypothesis. Nevertheless, existing information shows that many exogenous stimuli can induce apoptosis. The idea of a suicidal cell program has not been proved to be universal and it would be simpler to consider apoptosis a cellular response to stimuli. This concept implies the presence of target cells and appropriate levels of specific stimuli.

Does differentiation play any role in apoptosis? It is possible that apoptosis is the last stage of what is called "terminal differentiation." Differentiated cells could acquire receptors for apoptosis inducers. All those hypotheses could be tested in different experimental systems.

Mild injury sometimes results in apoptosis rather than necrosis. That type of injury may somehow mimic the apoptotic stimuli. (**Fig. 5**).

The most identifiable event in apoptosis is the internucleosomal fragmentation of DNA. This type of double-strand DNA damage probably cannot be repaired and by itself should be lethal. The nuclear and consequent cellular fragmentation are probably terminal or postmortem events, of great interest but not essential for understanding apoptosis.

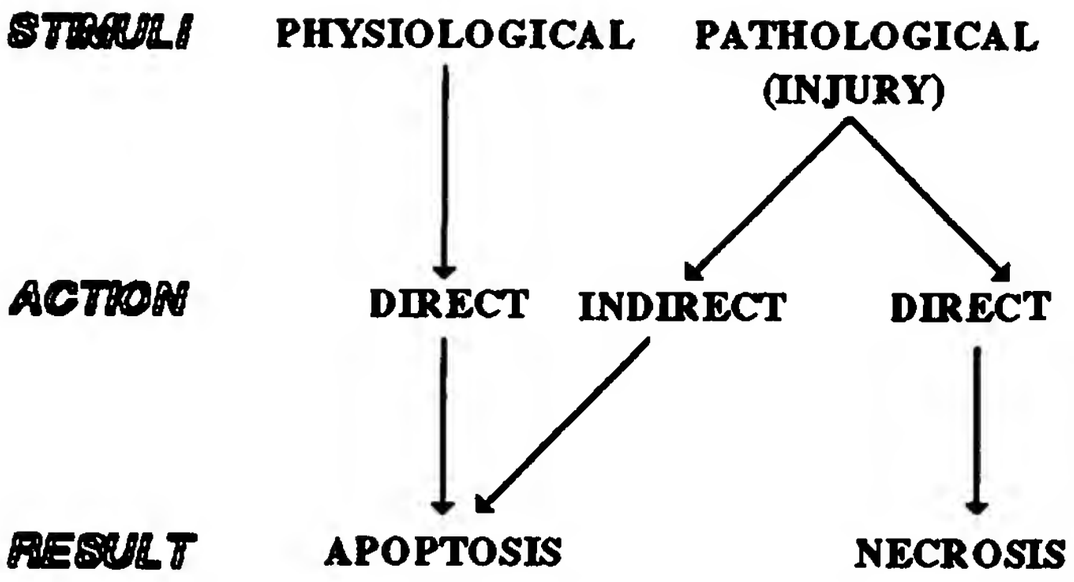

Figure 5. Apoptosis and necrosis stimuli. Physiological stimuli result in apoptosis whereas pathological stimuli result in necrosis. However, mild injury could result in apoptosis.

The internucleosomal fragmentation is the result of endonuclease (or endonucleases) action. It is possible that this enzyme (or enzymes) may be activated or that there is a need for its de novo synthesis. Changes in chromatin, which may result in exposing sites for endonuclease (or endonucleases) action, should be considered. It is also feasible that increases in endonuclease activity and chromatin changes may take place.

The existence of intracellular inducers or repressors of apoptosis for either of them has been gathered using RNA or protein synthesis inhibitors, which is suggestive but not definitive. It is possible that both of them may exist and interact, and the discrepancy in the experimental evidence may be due to differential half-life of mRNAs and/or proteins.

The general role of apoptosis appears to be crucial in development and growth regulation. It is clear that derangement in its regulation in development could result in structural and functional abnormalities, whereas lack of its tight regulation in growth could result in tumors. On the other hand, the fact that apoptosis is present in tumors suggests that its induction could be used as a therapy.

In summary, apoptosis appears to be a universal form of cell death that plays an important role in attaining and maintaining a normal organism. We know how to trigger it and what the final lethal events are, but there is a big knowledge gap in between, which is the target of active research. FJ

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